P/00/008b 12/11/91
Section 29 (1)
Regulation 3.1 (2)

AUSTRALIA

Patents Act 1990

NOTICE OF ENTITLEMENT

(To be filed before acceptance)

We, **Gesellschaft für Biotechnologische Forschung mbH (GBF)**, of Mascheroder Weg 1, D-38124 Braunschweig, Germany, being the applicant in respect of Application No. 36282/93 state the following:-

The Person nominated for the grant of the patent has entitlement from the actual inventors by assignment.

The person nominated for the grant of the patent is the applicant of the applications listed in the declaration under Article 8 of the PCT.

The basic applications listed on the request form are the first applications made in a Convention country in respect of the invention.

By our Patent Attorneys,
WATERMARK PATENT & TRADEMARK ATTORNEYS

.....

15 April 1996

Louis C. Gebhardt
Registered Patent Attorney

AU9336282

(12) PATENT ABRIDGMENT (11) Documen: No. AU-B-36282/93
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 672388

(54) Title
HYBRID DNA THAT CODES FOR A SUBUNIT OF A BACTERIAL TOXIN WITH A HEMOLYSIN

International Patent Classification(s)
(51)[5] C12N 015/62 A61K 039/02 C07K 015/04 A61K 039/112

(21) Application No. : 36282/93 (22) Application Date : 12.02.93

(87) PCT Publication Number : WO93/16186

(30) Priority Data

(31) Number	(32) Date	(33) Country
4204737	17.02.92	DE GERMANY
4219696	16.06.92	DE GERMANY

(43) Publication Date : 03.09.93

(44) Publication Date of Accepted Application : 03.10.96

(71) Applicant(s)
GESELLSCHAFT FUR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF)

(72) Inventor(s)
HIMANSHU BRAHMBHATT; GUO-FU SU; JURGEN WEHLAND; KENNETH N. TIMMIS

(74) Attorney or Agent
WATERMARK PATENT & TRADEMARK ATTORNEYS , Locked Bag 5, HAWTHORN VIC 3122

(57) Claim

1. Hybrid DNA, characterized in that

(a) a DNA structure coding for a subunit B of a Shiga toxin or Shiga-like toxins as a bacterial toxin, is fused with a DNA structure coding for the C-terminus of a HlyA (haemolysin) fragment and

(b) that a constitutive promotor or a promotor which can be induced under in vivo conditions for the expression in a Salmonella strain is provided in front of both coding DNA structures.

2. Hybrid DNA according to claim 1, characterized in that the bacterial toxin is SLT-II or SLT-IIv.

PCT NUMBER PCT/EP93/00348

AU9336282

INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

(51) Internationale Patentklassifikation 5 :
C12N 15/62, 15/74, C07K 7/00
C07K 7/10, A61K 39/02

A1

(11) Internationale Veröffentlichungsnummer: WO 93/16186

(43) Internationales Veröffentlichungsdatum: 19. August 1993 (19.08.93)

(21) Internationales Aktenzeichen: PCT/EP93/00348

(22) Internationales Anmeldedatum: 12. Februar 1993 (12.02.93)

(30) Prioritätsdaten:
P 42 04 737.4 17. Februar 1992 (17.02.92) DE
P 42 19 696.5 16. Juni 1992 (16.06.92) DE

(71) Anmelder *(für alle Bestimmungsstaaten ausser US)*: GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF) [DE/DE]; Mascheroder Weg 1, D-3300 Braunschweig (DE).

(72) Erfinder; und

(75) Erfinder/Anmelder *(nur für US)* : BRAHMBHATT, Himanshu [IN/DE]; SU, Guo-fu [CN/DE]; WEHLAND, Jürgen [DE/DE]; TIMMIS, Kenneth, N. [GB/DE]; Mascheroder Weg 1, D-3300 Braunschweig (DE).

(74) Anwälte: BOETERS, Hans, D. usw. ; Boeters & Bauer, Bereiteranger 15, D-8000 München 90 (DE).

(81) Bestimmungsstaaten: AU, JP, US, europäisches Patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Veröffentlicht
Mit internationalem Recherchenbericht.
Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist. Veröffentlichung wird wiederholt falls Änderungen eintreffen.

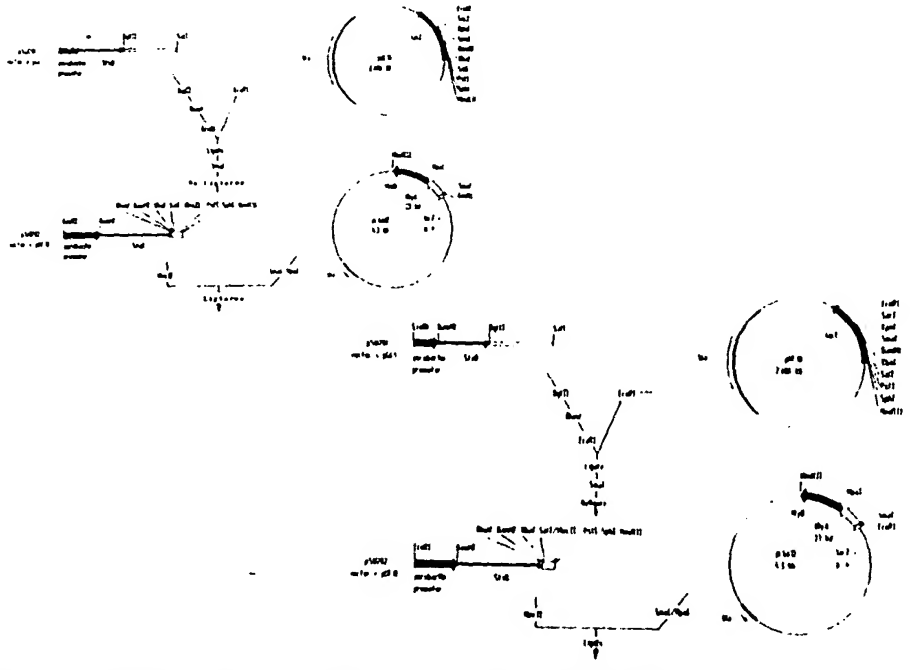
672388

(54) Title: HYBRID DNA THA ODES FOR A SUBUNIT OF A BACTERIAL TOXIN WITH A HEMOLYSIN

(54) Bezeichnung: HYBRID-DNA, DIE EINE UNTEREINHEIT EINES BAKTERIELLEN TOXINS MIT EINER HAEMOLYSIN KODIERT

(57) Abstract

A hybrid DNA is characterized in that a DNA structure that codes for a subunit of a bacterial toxin is fused with a HlyA (hemolysin)-coding DNA structure or its C-terminal fragment. Also disclosed are plasmides, host strains, oligohybridpeptides and vaccines obtained by use of said hybrid DNA.



(57) Zusammenfassung

Die Erfindung betrifft Hybrid-DNA, die dadurch gekennzeichnet ist, daß eine DNA-Struktur, die eine Untereinheit eines bakteriellen Toxins kodiert, mit einer HlyA (Haemolysin) kodierenden DAN-Struktur bzw. dessen C-terminalem Fragment fusioniert ist, ferner betrifft die Erfindung Plasmide, Wirtsstämme, Oligohybridpeptide und Impfstoffe unter Verwendung der genannten Hybrid-DNA.

Bacillary dysentery, caused by species of Shigella, is an invasive disease of the colonic mucosa that is limited to humans and certain other primates. *Shigella dysenteriae* 1 (Shiga's bacillus) causes the most severe form of the disease and can lead to severe complications like the haemolytic uraemic syndrome (H.U.S. which comprises haemolytic anaemia, thrombocytopenia and acute renal failure), leukemoid reactions and sepsis, particularly in infants. It is presently responsible for a major pandemic afflicting the Indian subcontinent, parts of Africa, South East Asia, China and parts of Latin America.

One of the distinguishing features of *S. dysenteriae* 1 is its production of large quantaties of Shiga toxin, one of the most potent cytotoxins known. Although Shiga toxin kills a wide variety of cell types, it is particularly active on vascular endothelial cells, which probably accounts for the severe complications associated with infections by Shiga's bacillus. Anti-dysentery vaccines should thus provide protection not only against the organism itself but also against Shiga toxin.

Shiga-like toxins also play a role in other infections of man and animals. Some serotypes of *Escherichia coli* such as O157:H7, produce haemorrhagic colitis, an afebrile watery diarrhoea that progresses to bloody diarrhoea, and in experimental animals, the typical symptoms of haemorrhagic colitis are produced by administration of purified toxin alone. Frequently associated with haemorrhagic colitis are severe complications such as H.U.S. which are also associated with Shiga bacillus infections. Shiga-like toxing producing *E. coli* serotypes also produce serious diseases, such as edema disease of pigs, in farm animals which lead to substantial economic losses.

There is thus an urgent need to develop vaccines for both human and animal applications against Shiga and related toxins.

Shiga and Shiga-like toxins are bipartite molecules consisting of two distinct types of subunit: the A-subunit (Mr=32,000) and the B-subunit (Mr=7,700) which associate noncovalently with an apparent subunit stoichiometry of one A-chain and five B-chains. The A-subunit is toxic to eucaryotic cells and the B-subunit has a receptor binding site which permits uptake of the toxin into the cells. Therefore an effective mucosal and serum anti-toxin immune response directed against the B-subunit should prevent the entry of toxin into host intestinal epithelial cells and vascular endothelial cells thereby eliminating the severity of the disease.

However, obtaining high levels of expression of foreign polypeptides in vaccine strains suffers from several drawbacks e.g.
(i) fusion of heterologous polypeptides to carrier molecules, such as the LamB protein, is often difficult because there is a limit on the size of the passenger polypeptide that can be stably inserted (Charbit et al. 1988; Guo-fu Su et al. 1992),
(ii) high level expression of heterologous polypeptides in the cytoplasm often results in the degradation of the protein and formation of inclusion bodies,
(iii) fusion of foreign polypeptides to carrier molecules such as ß-galactosidase to obtain expression in the cytoplasm also often results in toxicity to the cell making the vaccine strains sick.

There is therefore a need to develop suitable expression systems where large heterologous polypeptide can be stably expressed at high levels without forming inclusion bodies or resulting in toxicity to the vaccine strains.

Recently, a genetic system has been developed to export passenger proteins from *E. coli* to the medium via fusion to the 23 kD C-terminal signal domain of haemolysin (HlyA) (Mackman et al. 1987). The haemolysin genes are organized in an operon (hly C, A, B, D) and the HlyC protein is involved in post-translational activation of the 107 kD HlyA protein to its active form. The export mechanism includes a specific secretion signal within the last 50 C-terminal amino acids of HlyA and a membrane bound translocation complex, composed of HlyB and HlyD (Wagner et al. 1983), and at least one host protein, the minor outer membrane protein TolC (Wandersman and Delepelaire 1990), which transports the HlyA molecule directly to the medium without periplasmic intermediate (Gray et al. 1986; Koronakis et al. 1989).

In the following this art is explained in greater detail. In order to develop oral vaccines against infections caused by *Shigella dysenteriae* 1, hybrid attenuated Salmonella strains expressing the O-antigen of *S. dysenteriae* 1 have been constructed (Mills et al. 1988; Mills and Timmis 1988) with the aim to stimulate O-antigen specific, localized mucosal immune responses. A necessary additional component in the hybrid vaccine strains is the inclusion of suitably expresssed Shiga toxin B-subunit to elicit immune responses that will neutralize the biological activity of the toxin to reduce the severity of the disease.

The expression of foreign polypeptides fused to carrier molecules, such as the LamB protein, suffers from a drawback that there is a limit on the size of the passenger polypeptide that can be stably inserted (Charbit et al. 1988; Guo-fu Su et al. 1992). For larger polypeptides alternative approaches can be used such as fusion to the N-terminal region of ß-galactosidase (Jakob et al. 1985; Brown et al. 1987; Guo-fu Su et al. 1992).

Recently, a genetic system has been developed to export passenger proteins from *E. coli* to the medium via fusion to the 23 kD C-terminal haemolysin (HlyA) signal domain (Mackman et al. 1987) with the aim to purify recombinant proteins.

The cytotoxin, haemolysin, is secreted by uropathogenic strains of *Escherichia coli* (Welch et al. 1981) and is translocated into the medium without the aid of an N-terminal signal, secA, secY route (Felmlee et al. 1985; Blight et al. 1990). The haemolysin genes are organized in an operon (hly C, A B, D) and the HlyC protein is involved in post-translational activation of the 107 kD HlyA protein to its cytotoxic form. The export mechanism includes a specific secretion signal within the last 50 C-terminal amino acids of HlyA and a membrane bound translocation complex, composed of HlyB and HlyD (Wagner et al. 1983), and at least one host protein, the minor outer membrane protein TolC (Wandersman and Delepelaire 1990) which transports the HlyA molecule directly to the medium without a periplasmic intermediate (Gray et al. 1986; Koronakis et al. 1989).

According to the invention the Shiga toxin B-subunit has been fused to the 23 kd C-terminus of *E. coli* haemolysin A (HlyA) and it has been found that the fusion protein is exported from attenuated antigen carrier strain of *Salmonella typhimurium* aroA-(SL3261). The expression of the gene fusion has been studied under the control of a modified synthetic ß-lactamase promoter (constitutive expression) and under the in vivo inducible aerobactin promoter. Oral and intra-peritoneal immunization of mice with the hybrid *Salmonella typhimurium* aroA-(SL3261) strains resulted in the stimulation of significant B-subunit-specific mucosal and serum antibody responses.

This is the first report where a candidate antigen has been exported from antigen carrier vaccine strain of Salmonella to elicit antigen-specific immune responses. The system has the following advantages;
(i) large polypeptides can be fused to the haemolysin export machinery so long as the heterologous polypeptide does not encode "stop transfer" sequences,
(ii) since the fusion protein does not accumulate within the cells, reasonably high levels of expression can be achieved without resulting in toxicity to the vaccine strain.

This system is therefore appropriate for expression of Shiga-like toxins such as SLT-II and SLT-IIv to elicit strong immune responses against their receptor binding B-subunits, and against other bacterial toxins of mucosal pathogens.

According the the invention the Shiga toxin B-subunit protein has been fused to the 23 kD C-terminus of HlyA to export the B-subunit/HlyA fusion protein to the extracellular medium. These chimeric proteins have been expressed in *Salmonella typhimurium* aroA- antigen carrier vaccine strain SL3261 (Hoseith and Stocker 1981) for use in oral and intra-peritoneal immunizations to determine if B-subunit specific immune responses could be stimulated.

The Shiga-toxin B-subunit has been fused to the 23 kD C-terminus of *E. coli* haemolysin A (HlyA) and exported from attenuated antigen carrier strain of *Salmonella typhimurium* aroA$^-$ (SL3261) strain. The expression of the gene fusion has been studied under the control of a modified synthetic ß-lactamase promoter (constitutive expression) and under the in vivo inducible aerobactin promoter. The effect of plasmid copy number has also been analysed by using medium copy and high copy plasmid vectors. Stimulation of B-subunit specific antibody responses in

mice after oral and i.p. immunization with hybrid SL3261 strains has been carried out to study three different modes of B-subunit expression i.e.
(i) high-level cytoplasmic expression of B-subunit,
(ii) B-subunit/ß-galactosidase fusion protein expressed cytoplasmically and
(iii) B-subunit/HlyA (23 kD C-terminus) fusion protein exported into the extracellular environment.

According to the invention the expression of the Shiga toxin B-subunit/Haemolysin A (C-terminus) fusion proteins has been studied under four different conditions;
(a) where the stxB/C-T:hlyA gene fusion is under the control of an in vivo inducible aerobactin promoter carried either on plasmid vector pBR322 (copy number of approximately 30-40/cell/generation) or
(b) on plasmid pUC18 (copy number of approximately 70/cell/generation),
(c) the stxB/C-T:hlyA fusion under the control of a constitutive, moderate level expression, modified synthetic ß-lactamase promoter and the fusions are also expressed either from plasmids pBR322 or
(d) pUC18. This permitted a study of promoter strength and plasmid copy number on the stability of expression of the StxB/C-T:HlyA fusion protein.
(e) An additional comparative analysis was also conducted where the B-subunit was expressed at high levels in the cytoplasm and as a ß-galactosidase fusion protein also expressed in the cytoplasm.

Earlier studies using the haemolysin export machinery to secrete heterologous polypeptides were conducted in *E. coli* and it was not known whether such fusion proteins could also be exported from *Salmonella typhimurium*. Expression studies according to the

invention were simultaneously carried out both in *E. coli* K-12 and antigen carrier vaccine strain *S. typhimurium* aroA- SL3261.

The comparative analysis showed that the fusion proteins could be exported into the medium from both *E. coli* K-12 and *S. typhimurium* aroA- strains suggesting that the haemolysin export machinery was functional in *S. typhimurium*. The pUC18 based recombinant plasmids however proved toxic to the cells and some degradation of the fusion protein was observed. The bacterial cells harbouring the plasmids could not be grown to a cell density of greater than 10^7 live cells/ml. On the other hand, the pBR322 based plasmids were stable and there was no evidence of degradation of the fusion protein and the live cell density of 10^9 cells/ml was easily obtained. In earlier studies (Guo-fu Su et al. 1992), where various regions of the Shiga toxin B-subunit were fused into the bacterial sell surface exposed loop of the LamB protein, it was observed that only short B-subunit polypetpides could be stably expressed using LamB as a carrier molecule. The complete B-subunit (69 amino acids) fused into the LamB expression system proved toxic to the host bacteria and formed large intracellular aggregates (Guo-fu Su et al. 1992). The haemolysin export machinery therefore appears suitable when larger polypeptides need to be expressed as fusion proteins so long as the inserted polypeptide does not have any "stop transfer" sequences.

The hybrid *S. typhimurium* aroA- strains expressing B-subunit as
(i) cytoplasmic protein,
(ii) fused to ß-galactosidase and expressed cytoplasmically
(iii) fused to HlyA (C-terminus) and exported into the extracellular environment were used for oral and i.p. immunization in mice to analyse which expression system proved most succesful in stimulation of B-subunit specific antibody responses. The results show that significant B-subunit antibody responses could be elicited using any of the three systems.

However high level expression of candidate antigen appears to be deleterious to the host bacteria and therefore moderate level expression appears to be more suitable. Constitutive expression such as the synthetic ß-lactamase promoter or in-vivo inducible system such as the aerobactin promoter have provided better results in terms of the stability of the host bacteria.

The serum responses in general were higher than mucosal antibody responses. One possible reason for this observation could be that the recovery of antibodies from the intestine is not efficient. The B-subunit/ß-galactosidase fusion proteins elicited significantly higher levels of ß-galactosidase responses presumably reflecting higher epitope density in the much larger ß-galactosidase polypeptide (107 kD) as compared to the B-subunit (7.7 kD).

In the following the invention is explained by means of figures and experimental data in greater detail.

Figure Legends.

Fig. 1

Plasmid constructions for the expression of StxB/C-T:HlyA fusion proteins. For convenience, only the relevant parts of the insert of plasmids are shown.

Fig. 2

Western-Blott analysis of expression of Shiga toxin B-subunit/haemolysin A (C-terminus) fusion proteins. The B-subunit regions of the fusion proteins are revealed using the B-subunit specific monoclonal antibody StxBMb1.

(A) Lane 1, *E. coli* JM 101/pLG612+pLG575 (whole cell extract); lane 2, culture supernatant of cells from lane 1; lane 3, JM101/pSU204+pLG575 (aerobactin promoter/stxB/C-T:hlyA/pBR322; whole cell extract); lane 4, culture supernatant of cells from lane 3; lane 5, JM101/pSU206+pLG575 (MS ß-lactamase promoter/stxB/C-T:hlyA/pBR322; whole cell extract); lane 6, culture supernatant of cells from lane 5.
(B) Same as Fig. 2A except that the host bacterium is *Salmonella typhimurium* aroA⁻SL3261.
(C) Lane 1, SL3261/pLG612+pLG575 (whole cell extract); lane 2, culture supernatant of cells from lane 1; lane 3, SL3261/pSU203+pLG575 (aerobactin promoter/stxB/C-T:hlyA/pUC18; whole cell extract); lane 4, culture supernatant of cells from lane 3; lane 5, SL3261/pSU205+pLG575 (MS ß-lactamase promoter/stxB/C-T:hlyA/pUC18; whole cell extract); lane 6, culture supernatant of cells from lane 5.

Fig. 3

Mucosal and serum antibody responses in mice after oral and i.p. immunization with various hybrid *S. typhimurium* aroA⁻strains (SL3261).

Fig. 3.1.

B-subunit responses after immunizations with SL3261/pSU108 (high-level cytoplasmic expression of B-subunit) and SL3261/pJLA503 (negative control).

Fig. 3.2.

B-subunit and ß-galactosidase antibody responses after immunizations with SL3261/pSU207 (B-subunit/ß-galactosidase fusion protein expressed cytoplasmically; expression under the control of aerobactin promoter) and SL3261/pcon1 (plasmid vector

expressing lacZ under the control of aerobactin promoter; negative control for B-subunit responses).

Fig. 3 3.

B-subunit antibody responses after immunization with SL3261/pLG575/pSU204 (expressing stxB/C-T::hlyA under the control of aerobactin promoter), SL3261/pLG575/pSU206 (expressing stxB/C-T::hylA under the control of MS-ß-lactamase promoter) and SL3261/pLG575/pLG612 (expressing C-T::hlyA under the control of lac promoter; negative control for B-subunit responses).

MATERIALS AND METHODS.

Bacterial strains, plasmids and media.

E. coli strain JM101 (F-, traD36, lacI^q, (lacZ)M15, proAB, supE, thi, lac-proAB; Yanisch-Perron et al. 1985) was used as the recipient for all recombinant plasmids. *Salmonella typhimurium* aroA- mutant SL3261 (Hoseith and Stocker 1981) and the restriction negative *S. typhimurium* strain SL5283 were kindly provided by B.A.D. Stocker (Stanford Univ. Sch. of Medicine, USA). Plasmids pLG612 (carries the 23 kD C-terminus of hlyA and part of the N-terminus of hlyB under the control of the lac promoter inducible with IPTG) and pLG575 (chloramphenicol resistant plasmid carrying the hlyB and hlyD genes cloned into the tet resistance gene of plasmid pACYC184; Mackman et al. 1985) were kindly provided by Dr. Holland (Univ. of Leicester, U.K.). Plasmid pSU201 (Guo-fu Su et al. 1992) carries the stxB gene under the control of the aerobactin promoter and plasmids pBR322 (Bolivar et al. 1977) and pUC18 (Yanisch-Perron et al. 1985) were used as general purpose cloning vectors. The following plasmids have been described by Guo-fu Su and

colleagues (1992); (i) plasmid pSU108 (carrying stxB gene under the control of lambda P_L and P_R promoters which are thermoinducible), (ii) plasmid pJLA503 (high level expression plasmid; Schauder et al. 1987), (iii) plasmid pSU207 (carrying stxB/lacZ gene fusion under the control of aerobactin promoter), (iv) plasmid pcon1 (carries the lacZ gene under the control of aerobactin promoter; Lorenzo et al. 1987).
Luria broth and Luria agar (Miller 1972) were used as complete media for the routine growth of all strains and where appropriate, bacterial growth media were supplemented with antibiotics ampillicin (100 μg/ml) or chloramphenicol (30 μg/ml). Restriction endonucleases, T4 DNA ligase, DNA polymerase (Klenow enzyme) and all other enzymes were purchased either from Boehringer GmbH (Mannheim, Germany) or from New England Biolabs, Inc. Beverly, Mass.; and were used as recommended by the manufacturer.

DNA manipulations.

DNA preparation and genetic manipulations were carried out according to standard protocols (Maniatis et al. 1982) and plasmid DNA transformation of bacterial cells was performed as described by Hanahan (1983).

SDS-polyacrylamide gel electrophoresis.

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Schaegger and Jagow (1987) SDS-PAGE prestained molecular weight markers were either from BIO-Rad (in kD; 97.4, 66.2, 45.0, 31.0, 21.5, 14.4) or from Sigma (in kD; 84.0, 58.0, 48.5, 36.5, 26.6) as metioned in the figure legends.

Western blotting of whole cell extracts and culture supernatant fractions.

Bacterial cells from 1 ml of over-night culture were collected by centrifugation and to the supernatant fraction (approximately 900 µl), 100 µl of 100 % TCA (Tri-chloro acetic acid) was added to precipitate the extracellular proteins. After thorough mixing, the sample was incubated at 4° C for 30 min, centrifuged for 15 min and the supernatant was discarded. The protein pellet (culture supernatant fraction) was resuspended in 20 µl sample buffer (16 µl 1M Tris-base, 2 µl 10x cracking buffer [1x cracking buffer: 60 mM Tris-HCl pH 6.8, 1 % SDS, 1 % 2-mercaptoethanol, 10 % glycerol, 0,01 mg bromophenol blue], 2 µl glycerol). The bacterial pellet (whole cell fraction) was resuspended in 50 µl sample buffer. The supernatant and whole cell fractions were heated to 100 °C for 10 min. before loading 20 µl samples on SDS-PAGE. After electrophoresis the gels were blotted onto nitrocellulose and the Shiga toxin B-subunit/HlyA fusion proteins were revealed using the StxBMb1 B-subunit specific monoclonal antibody (Guo-fu Su et al. 1992).

Immunization and collection of sera and intestinal washings.

Oral and i.p. immunizations of 8-10 week old female BALB/c mice (four mice per immunization) were carried out essentially as described by Guo-fu Su and colleagues (1992). Innocula for immunization were prepared as follows.

Overnight culture of *S. typhimurium* aroA$^-$ strain SL3261 either carrying plasmid pSU108 or pJLA503 (negative control) were freshly cultivated in Luria broth containing ampicillin until OD_{600} reached 0.7. The cells were shifted to 42 °C for 45 min to induce the lambda P_L and P_R promoters. The cultures were washed

twice in sterile normal saline and resuspended in an appropriate volume of saline to obtain a final concentration of 10^{11} CFU/ml. 0.1 ml cell suspension was used for oral immunization. Cells were further diluted to 10^6 CFU/ml and 0.1 ml cells were used for i.p. immunization.

SL3261 strains carrying either plasmids pcon1 (negative control) or pSU207 were also grown as above until OD_{600} reached 0.3. 2,2'-bipyridyl was added (final concentration of 100 μM; for induction of the aerobactin promoter) and cells were grown further for 3 hr. The cultures were washed, resuspended and used for immunization as mentioned above.

SL3261/pLG575 strains carrying either plasmids pLG612 (negative control), pSU204 or pSU206 were grown as above in Luria broth supplemented with ampicillin and chloramphenicol until OD_{600} reached 1.0 (for plasmid pSU206) and 0.3 (for plasmid pLG612 and pSU204). Cells carrying plasmid pLG612 were induced with IPTG (final concentration 1 mM) and cells carrying plasmid pSU204 were induced with 2,2'-bipyridyl (final concentration 100 μM). SL3261/pLG575/pLG612 cultures were grown further for 45 minutes and SL3261/pLG575/pSU204 cultures were grown further for 3 hrs. The cells were washed, resuspended and used for immunization as stated above.

ELISA.

Samples for ELISA were serially diluted in phosphate-buffered saline (pH 7.2). Anti-B-subunit and anti-ß-galactosidase determinations were carried out by pre-coating microtiter plates with 1 μg of purified B-subunit (Guo-fu Su et al. 1992) and 1 μg purified ß-galactosidase (purchased from Sigma Chemical Co.) respectively. Serum IgG + IgM was determined using peroxidase conjugated goat anti-mouse IgG + IgM (purchased from Dianova)

and mucosal IgA was determined using peroxidase conjugated goat anti-mouse IgA (obtained from Southern Biotechnology Inc.). The results were read using the Bio-Rad Microplate Reader (Model 3550).

Plasmid construction for expression of HlyA (C-terminus)/B-subunit fusion proteins.

The EcoRI/BglII fragment from phagemid pSU201 (Guo-fu Su et al. 1992) carrying stxB under the control of the aerobactin promoter was subcloned into the EcoRI/SmaI sites of plasmid pUC18 to give plasmid pSU202 (as shown in Fig. 1). The C-terminal 23 kD region of hlyA (designated C-T:hlyA) and approximately 50 bp of the N-terminal region of hlyB, carried on a SmaI/HpaI fragment was inserted into the HincII site of plasmid pSU202. The resulting plasmid, pSU203 (Fig. 1) carries the stxB gene fused in-frame with C-T:hlyA. The EcoRI/HINDIII fragment of plasmid pSU203 carrying the aerobactin promoter stxB/C-T:hlyA cassette was subcloned into the EcoRI/HINDIII sites of plasmid pBR322 to give plasmid pSU204 (Fig. 1).

The EcoRI/BamHI fragment of plasmid pSU203 which carries the aerobactin promoter was replaced with the modified synthetic ß-lactamase promoter (designated MS ß-lactamase promoter; for details, see Guo-fu Su et al. 1992). This construction resulted in plasmid pSU205 (Fig. 1) which carries an EcoRI/HINDIII cassette comprising of the MS ß-lactamase promoter/stxB/C-T:hlyA region which could be inserted into the EcoRI/HindIII sites of plasmid pBR322 to give plasmid pSU206 (Fig. 1).

Plasmids pSU203 and pSU204 (carrying the aerobactin promoter/ stxB/C-T:hlyA cassette in pUC18 and pBR322 vectors respectively), and plasmids pSU205 and pSU206 (carrying MS ß-lactamase promoter/stxB/C-T:hlyA cassette in pUC18 and pBR322

vectors respectively), were transformed into *E. coli* K-12 and *S. typhimurium* aroA$^-$ SL3261 strains carrying plasmid pLG575. Prior to transformation of *S. typhimurium* aroA$^-$ SL3261, the plasmids were first passaged through tne restriction negative *S. typhimurium* strain SL5283.

Western blotting analysis of StxB/C-T:HLyA fusion proteins in *E. coli* K-12 and *Salmonella typhimurium* aroA- strain SL3261. The pellet and supernatant fractions of the bacterial cells harbouring the recombinant and control plasmids were electrophoresed on SDS-PAGE and blotted onto nitrocellulose. The fusion proteins were revealed using the B-subunit specific monoclonal antibody, StxBMb1 (Fig. 2). The StxB/C-T:HlyA fusion protein was found to be exported into the extra-cellular environment in all the strains analysed (Figs. 2A, B and C, lanes 4 and 6). The StxB/C-T:HlyA fusion protein expressed by the aerobactin promoter is 7 kD larger than the same fusion protein expressed by the MS ß-lactamase promoter since the EcoRI/BamHI aerobactin promoter fragment in plasmid pSU201 (Fig.1) also carries a part of the iucA gene (the first gene of the aerobactin operon) which encodes a 7 kD polypeptide (Lorenzo et al. 1987).

The amount of the fusion protein expressed was much higher in pUC18 based plasmids as compared to pBR322 based vector (Compare Fig. 2B and C, lanes 4 and 6). However, the higher level of expression also led to some degradation of the fusion protein (Fig. 2C, lanes 4 and 6) and also proved toxic to the host cells since it was not possible to grow these bacteria to a live cell density of more than 10^7 cells/ml. The pBR322 based constructs were stable and the cells could be grown to 10^9 live cells/ml.

Analysis of B-subunit specific immune responses in mice after oral and intra-peritoneal immunization with hybrid *Salmonella typhimurium* aroA- strain SL3261.

Salmonella typhimurium aroA- (SL3261) strains expressing B-subunit in three different forms were analysed for the stimulation of B-subunit specific immune responses in mice after oral or i.p. immunization. The expression systems tested include;
(i) cytoplasmic high-level expression of B-subunit (plasmid pSU108; Guo-fu Su et al. 1992),
(ii) cytoplasmic expression of B-subunit/ß-galactosidase fusion protein (plasmid pSU207; Guo-fu Su et al. 1992),
(iii) B-subunit/C-T::HlyA fusion protein expressed either under the control of the aerobactin or the MS-ß-lactamase promotor (plasmids pSU204 and pSU206). These fusion proteins are exported into the extracellular space.

Intestinal fluid was analysed for anti-B-subunit mucosal IgA and serum IgG and IgM antibody responses while the serum from i.p. immunized mice was analysed for IgG and IgM antibody responses. The samples obtained after immunization with *S. typhimurium* aroA$^-$carrying plasmid pSU207 were also analysed for ß-galactosidase responses. The results (Figs. 3.1, 3.2 und 3.3) showed that in all cases significant B-subunit specific antibody responses were detected in both the serum and intestinal fluid. However, plasmid pSU108 carrying salmonellae were unstable presumably due to the high-level expression of the recombinant B-subunit. The serum antibody responses were in general higher than mucosal antibody responses. For plasmid pSU207 (Fig. 3.2) anti-ß-galactosidase antibody responses were significantly higher than B-subunit specific antibody responses.

Biological Material	Availability
DNA for HlyA (= Haemolysin	cf. pSU 204 = DSM 7045 or pSU 205 = DSM 7046
SLT-II (= Shiga Like Toxin II)	Stockbin et al., Infect. Immun., 50 (1985) 695-700 and 53 (1986) 135-140
SLT-IIv	loc. cit.
ß-lactamase-Promoter	cf. pSU 205 = DSM 7046
Aerobactin-Promoter	cf. pSU 204 = DSM 7045
pBR 322	Boehringer Mannheim
pUC 18	Boehringer Mannheim
S. t. aroA SL3261	Hosieth & Stocker, Nature, 291 (1981) 238-239

REFERENCES.

Blight MA, Holland IB (1990) Structure and function of haemolysin B, P-glycoprotein and other members of a novel family of membrane translocators. Molec. Microbiol. 4: 873-880.

Bolivar F, Rodriguez RL, Betlach MC, Boyer HW (1977) Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of plasmid pMB9, Gene 2: 75-93.

Brown A, Hormaeche CE, De Hormaeche RD, Winther M, Dougan G, Maskell DJ, Stocker BAD (1987) An attenuated aroA *Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned ß-galactosidase in mice. J. Infect. Dis. 155: 86-92.

Charbit A, Molla A, Saurin W, Hofnung M. (1988) Versatility of a vector to express foreign polypeptides at the surface of Gram-negative bacteria. Gene 70: 181-189.

Felmlee T, Pellet S, Lee E-Y, Welch R (1985) *Escherichia coli* haemolysin is released extracellularly without cleavage of a signal peptide. J. Bacteriol. 163: 88-93.

Gray L, Mackman N, Nicaud J-M, Holland IB (1986).....Mol. Gen. Genet. 205: 127-133.

Guo-fu su Su, Brahmbhatt HN, Wehland J, Rohde M, Timmis KN (1992) Construction of stable LamB/Shiga toxin B-subunit hybrids, analysis of expression in *Salmonella typhimurium* aroA-strains and stimulation of B-subunit specific mucosal and serum antibody responses: Potential anti-Shiga toxin component of dysentery vaccine.....

Hanahan D (1983) Studies in transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166: 577-580.

Hoiseth SK, Stocker B.A.D. (1981) Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature 291: 238-239.

Koronakis V, Kornakis E, Hughes C (1989) Isolation and analysis of the C-terminal signal directing the export of *Escherichia coli* haemolysin protein across both bacterial membranes. EMBO J. 8: 595-605.

Lorenzo VD, Wee S. Herrero M, Neilands JB (1987) Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (fur) repressor. J. Bacteriol. 169: 2624-2630.

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY.

Mackman N, Nicaud J-M, Gray L, Holland IB (1985) Genetical and functional organization of the *Escherichia coli* haemolysin determinant 2001. Mol. Gen. Genet. 201:282-288.

Mackman N, Baker K, Gray L, Haigh R, Nicaud J-M, Holland IB (1987) Release of a chimeric protein into the medium from *Escherichia coli* using the C-terminal secretion signal of haemolysin. EMBO J. 6:2835-2841.

Mills SD, Sekizaki T, Gonzales-Carrero MI, Timmis KN (1988) Analysis and genetic manipulation of Shigella virulence determinants for vaccine development. Vaccine 6: 116-122.

Mills SD, Timmis KN (1988) Genetics of O-antigen polysaccaride biosynthesis in Shigella and vaccine development. In: Cabello FC, Pruzzo C (Eds) Bacteria, Complement and the Phagocytic Cell. Springer Verlag, Berlin, Heidelberg, pp 21-39.

Schaegger H, Jagow GV (1987) Tricine-sodium Dodecyl Sulfate-Polyacrylamide Gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166: 368-379.

Schauder B, Bloecker H, Frank R, Mc Carthy JEG (1987) Inducible expression vectors incorporating the *Escherichia coli* atpE translation initiation region. Gene 52: 279-283.

Smith HW (1963) The haemolysins of *Escherichia coli*. J. Pathol. Bacteriol. 85: 197-211.

Wagner W, Vogel M, Goebel W (1983) Transport of haemolysin across the outer membrane of *Escherichia coli* requires two functions. J. Bacteriol. 154: 200-210.

Wandersman C, Delepelaire P (1990) TolC, and *Escherichia coli* outer membrane protein required for haemolysin secretion. Proc. Natl. Acad. Sci. USA 87: 4776-4780.

Welch RA, Patchen-Dellinger E, Minschew B, Falkow S (1981) Haemolysin contributes to virulence of extraintestinal *E. coli* infections. Nature 294: 665-667.

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Hybrid DNA, characterized in that

(a) a DNA structure coding for a subunit B of a Shiga toxin or Shiga-like toxins as a bacterial toxin, is fused with a DNA structure coding for the C-terminus of a HlyA (haemolysin) fragment and

(b) that a constitutive promotor or a promotor which can be induced under in vivo conditions for the expression in a Salmonella strain is provided in front of both coding DNA structures.

2. Hybrid DNA according to claim 1, characterized in that the bacterial toxin is SLT-II or SLT-IIv.

3. Hybrid DNA according to claim 1, characterized in that a promotor for the expression in S. typhimurium, for example S. t. aroA$^-$ SL3261, is provided in front of both coding DNA structures.

4. Hybrid DNA according to claim 3, characterized by a β-Lactamase promotor of the wild type or as a modification of the wild type, wherein the promotor can be provided synthetically or not.

5. Hybrid DNA according to claim 3, characterized by an Aerobactin promotor.

6. Plasmid, characterized by a hybrid DNA according to any one of the preceding claims.

7. Host strain, characterized by a plasmid according to claim 6, especially a Salmonella strain, especially S. typhimurium, for example S. t. aroA$^-$ SL3261.

8. Oligohybrid peptide, characterized by a subunit B of a Shiga toxin or Shiga-like toxin which has been fused with the C-terminal fragment of HlyA.

9. Vaccine, consisting of or comprising the oligohybrid peptide according to claim 8 together with pharmaceutically acceptable carriers and excipients.

DATED this 2nd day of August, 1996

Gesellschaft für Biotechnologische Forschung mbH (GBF)

WATERMARK PATENT & TRADEMARK ATTORNEYS
290 BURWOOD ROAD
HAWTHORN VICTORIA 3122
AUSTRALIA

AU3628293.WPC[DOC. 007] KJS/SI:ML

Fig. 1

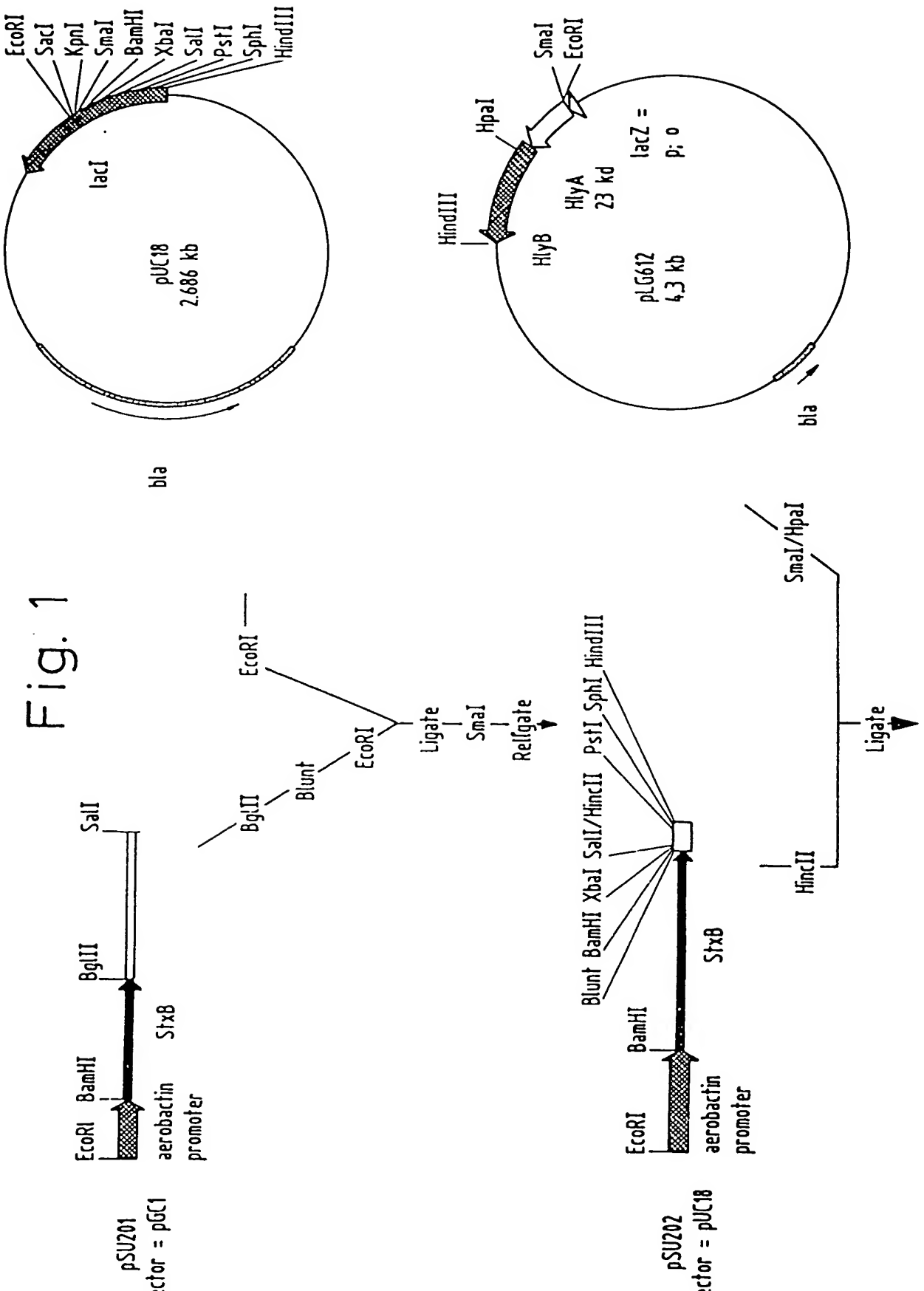

EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
lacI
pUC18
2.686 kb
bla
HindIII
HpaI
SmaI
EcoRI
HlyB
HlyA
23 kd
pLG612
4,3 kb
lacZ =
p; o
bla
pSU201
vector = pGC1
EcoRI
BamHI
BglII
SalI
aerobactin
promoter
StxB
BglII
Blunt
EcoRI
EcoRI
Ligate
SmaI
Relfgate
pSU202
vector = pUC18
EcoRI
BamHI
aerobactin
promoter
StxB
Blunt BamHI XbaI SalI/HincII PstI SphI HindIII
SmaI/HpaI
HincII
Ligate

Fig. 2

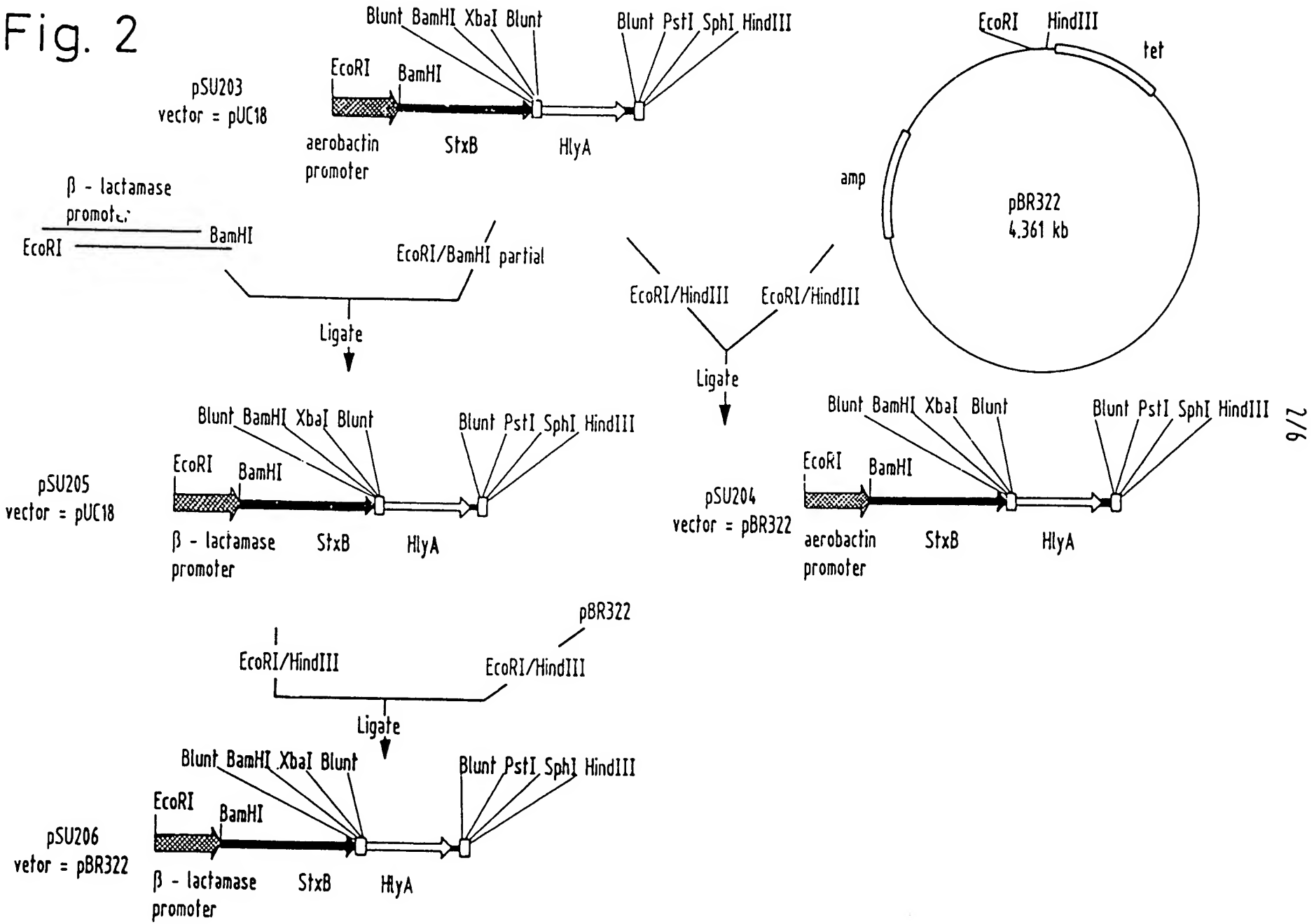

pSU203
vector = pUC18
EcoRI
BamHI
Blunt BamHI XbaI Blunt
Blunt PstI SphI HindIII
aerobactin promoter
StxB
HlyA
β - lactamase promoter
EcoRI
BamHI
EcoRI/BamHI partial
Ligate
pSU205
vector = pUC18
β - lactamase promoter
StxB
HlyA
EcoRI/HindIII
EcoRI/HindIII
Ligate
pSU204
vector = pBR322
aerobactin promoter
pBR322
EcoRI/HindIII
EcoRI/HindIII
Ligate
pSU206
vetor = pBR322
β - lactamase promoter
pBR322
4.361 kb
amp
tet

Fig. 3

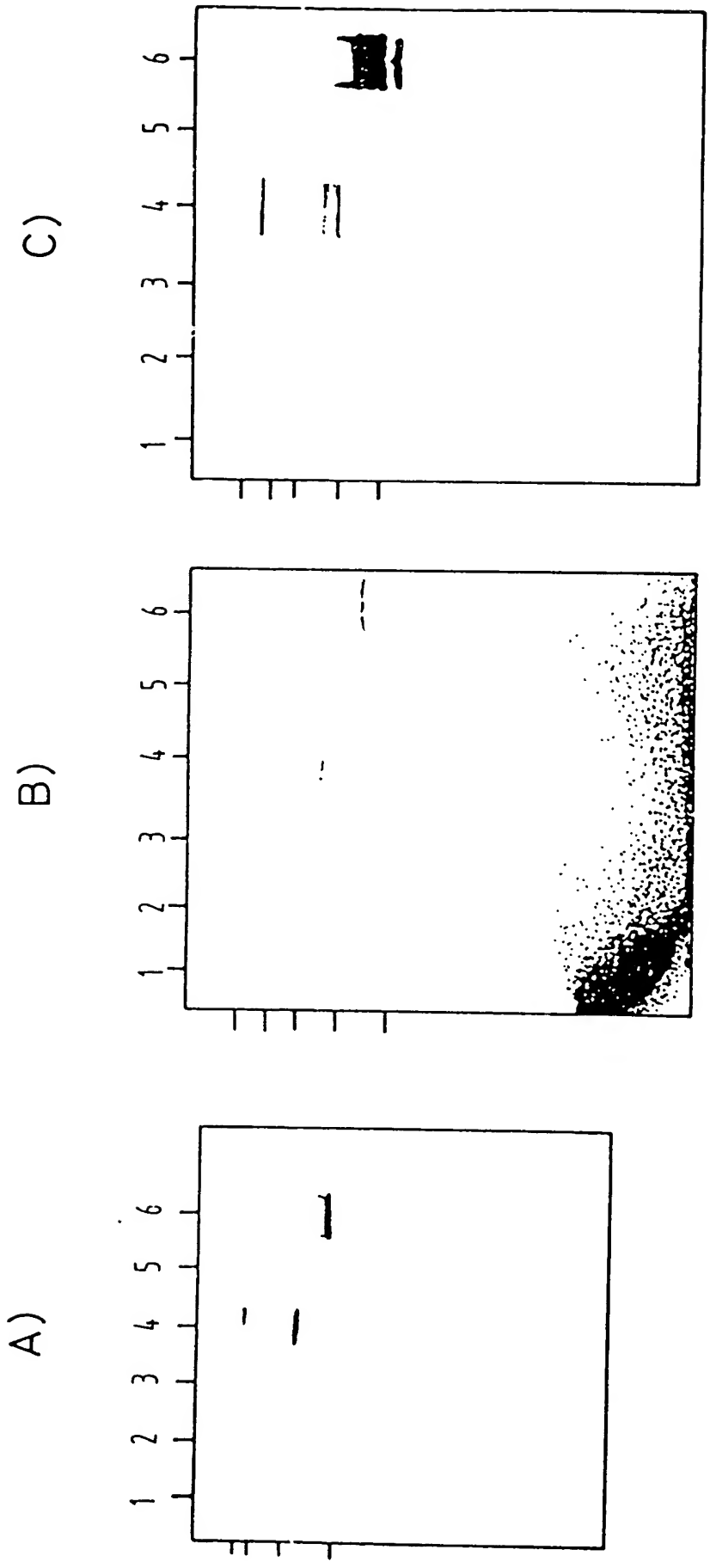


Fig. 4

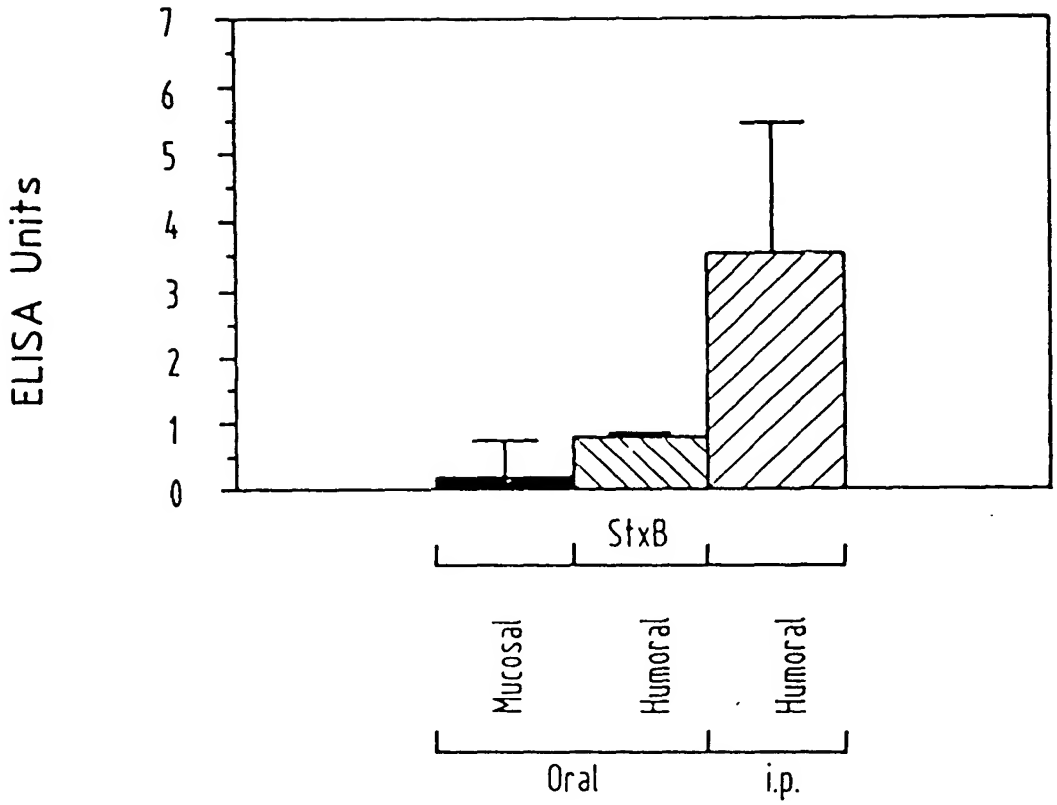

ELISA Units
7
6
5
4
3
2
1
0
StxB
Mucosal
Humoral
Humoral
Oral
i.p.
B-subunit/mucosal
B-subunit/Humoral
B-subunit/Humoral

Fig. 5

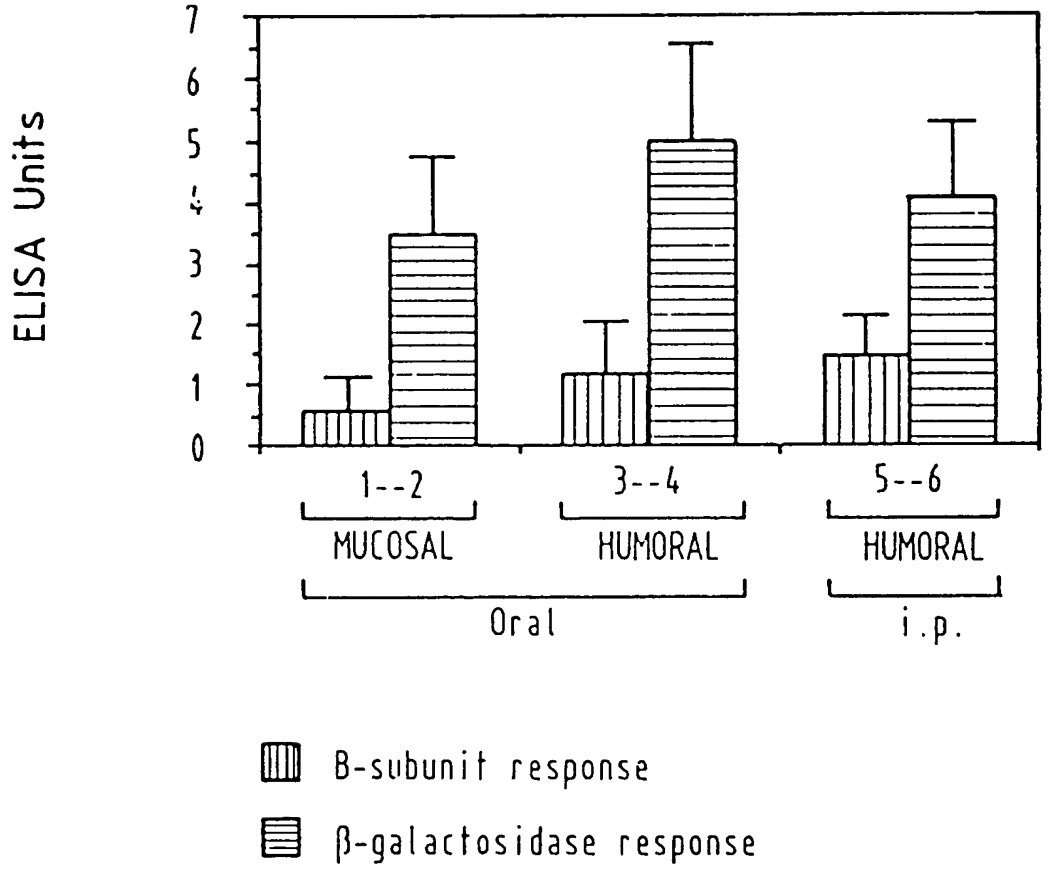
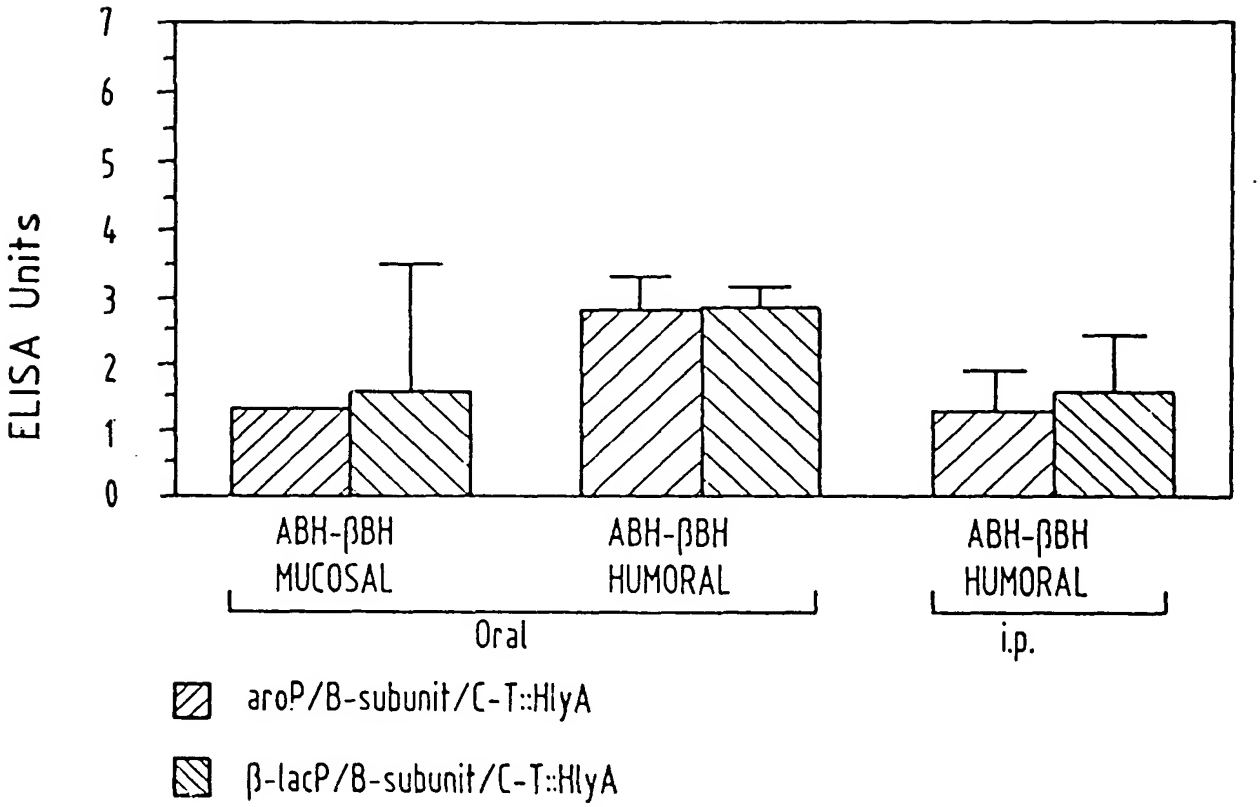

ELISA Units
7
6
5
4
3
2
1
0
1--2
3--4
5--6
MUCOSAL
HUMORAL
HUMORAL
Oral
i.p.
B-subunit response
β-galactosidase response

Fig. 6


ELISA Units
7
6
5
4
3
2
1
0
ABH-βBH MUCOSAL
ABH-βBH HUMORAL
ABH-βBH HUMORAL
Oral
i.p.
aroP/B-subunit/C-T::HlyA
β-lacP/B-subunit/C-T::HlyA

International application No
PCT/EP 93/00348

A CLASSIFICATION OF SUBJECT MATTER

Int.Cl.[5] C 12 N 15/62; C 12 N 15/74 ; C 07 K 7/00; C 07 K 7/10
A 61 K 39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.CL.[5] C 07 K; C 12 N; A 61 K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BACTERIOLOGY volume 174, No 1, January 1992, pages 291 - 297 DALTON R. MCWHINNEY ET AL:" Separable domains define target cell specificities of an RTX Hemolysin from Actinobacillus pleuropneumoniae" see abstract see page 291, left-hand column, paragraph 1 - right-hand column, paragraph 1 see page 294, left-hand column, paragraph 2 see page 293, right-hand column, paragraph 2 - page 194, left-hand column, paragraph 1 -- -/--	1,3,7,9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 May 1993 (18.05.93)	21 June 1993 (21.06.93)

Name and mailing address of the ISA	Authorized officer:
European Patent office Facsimile No	Telephone No

Form PCT/ISA/210 (second sheet) (July 1992)

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INFECTION AND IMMUNITY volume 59, No 11, November 1991, WASHINGTON US pages 4212 - 4220 CHRISTIANE FORESTIER ET AL: "Identification of RTX toxin target cell specificity domains by use of hybrid genes" see abstract see page 4212, left-hand column, paragraph 1 - right-hand column, paragraph 2, see page 4212, right-hand column, paragraph 3 see page 4214, right-hand column, paragraph 2 see page 4216, right-hand column, paragraph 3 --	1,7,9
A	EMBO JOURNAL volume 6, No 9, September 1987, EYNSHAM, OXFORD GB pages 2835 - 2841 N. MACKMAN ET AL." Release of a chimeric protein into the medium from Escherichia coli using the C-terminal secretion signal of haemolysin" cited in the application see page 2835, right-hand column, paragraph 2 - page 2836, right-hand column, paragraph 1 --------	1,3,5,7,9

I. KLASSIFIKATION DES ANMELDUNGSGEGENSTANDS (bei mehreren Klassifikationssymbolen sind alle anzugeben)[6]

Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC

Int.Kl. 5 C12N15/62; C12N15/74; C07K7/00; C07K7/10
A61K39/02

II. RECHERCHIERTE SACHGEBIETE

Recherchierter Mindestprüfstoff [7]

Klassifikationssytem	Klassifikationssymbole
Int.Kl. 5	C07K ; C12N ; A61K

Recherchierte nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Sachgebiete fallen [8]

III. EINSCHLAGIGE VEROFFENTLICHUNGEN [9]

Art.°	Kennzeichnung der Veröffentlichung [11], soweit erforderlich unter Angabe der maßgeblichen Teile [12]	Betr. Anspruch Nr.[13]
X	JOURNAL OF BACTERIOLOGY Bd. 174, Nr. 1, Januar 1992, Seiten 291 - 297 DALTON R. MCWHINNEY ET AL. 'Separable domains define target cell specificities of an RTX Hemolysin from Actinobacillus pleuropneumoniae' siehe Zusammenfassung siehe Seite 291, linke Spalte, Absatz 1 - rechte Spalte, Absatz 1 siehe Seite 292, linke Spalte, Absatz 2 siehe Seite 293, rechte Spalte, Absatz 2 - Se · 294, linke Spalte, Absatz 1 --- -/--	1,3,7,9

° Besondere Kategorie gegebenen Veröffentlichungen [10] :

"A" Veröffentlichung allgemeinen Stand der Technik definiert, aber n besonders bedeutsam anzusehen ist

"E" älteres Dokume jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden

"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

IV. BESCHEINIGUNG

1

Datum des Abschlusses der Internationalen Recherche	Absendedatum des Internationalen Recherchenberichts
18.MAI 1993	21 -06- 1993
Internationale Recherchenbehörde	**Unterschrift des bevollmächtigten Bediensteten**
EUROPAISCHES PATENTAMT	MONTERO LOPEZ B.

III. EINSCHLAGIGE VEROFFENTLICHUNGEN (Fortsetzung von Blatt 2)

Art *	Kennzeichnung der Veröffentlichung, soweit erforderlich unter Angabe der maßgeblichen Teile	Betr. Anspruch Nr.
X	INFECTION AND IMMUNITY Bd. 59, Nr. 11, November 1991, WASHINGTON US Seiten 4212 - 4220 CHRISTIANE FORESTIER ET AL. 'Identification of RTX toxin target cell specificity domains by use of hybrid genes' siehe Zusammenfassung siehe Seite 4212, linke Spalte, Absatz 1 - rechte Spalte, Absatz 2 siehe Seite 4212, rechte Spalte, Absatz 3 siehe Seite 4214, rechte Spalte, Absatz 2 siehe Seite 4216, rechte Spalte, Absatz 3 ---	1,7,9
A	EMBO JOURNAL Bd. 6, Nr. 9, September 1987, EYNSHAM, OXFORD GB Seiten 2835 - 2841 N. MACKMAN ET AL. 'Release of a chimeric protein into the medium from Escherichia coli using the C-terminal secretion signal of haemolysin' in der Anmeldung erwähnt siehe Seite 2835, rechte Spalte, Absatz 2 - Seite 2836, rechte Spalte, Absatz 1 -----	1,3,5,7, 9